Extraction of astaxanthin from *Haematococcus pluvialis* with hydrophobic deep eutectic solvents

Walter Pitacco,[a] Chiara Samorì,[a,b] Laura Pezzolesi,[c] Virgina Gori,[c] Matina Vagnoni,[a] Paola Galletti [a,b]

[a]*Dipartimento di Chimica "Giacomo Ciamician", Università di Bologna, Via Selmi, 2, 40126, Bologna (Italy)*
[b] *CIRI-FRAME, University of Bologna, via Sant'Alberto 163, Ravenna, Italy*
[c] *Dipartimento di Scienze Biologiche, Geologiche ed Ambientali, University of Bologna, via Sant'Alberto 163, Ravenna, Italy*

**walter.pitacco2@unibo.it*

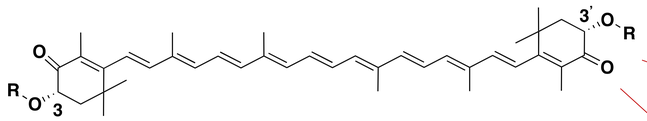
H. Pluvialis **is a biflagellate unicellular green alga categorized under the class of Chlorophyceae**

The cultivation of *H. pluvialis* consists of two major phases, namely green motile stage and red non-motile stage.

H. pluvialis **reacts to stressful environmental conditions (e.g., light, temperature and salt concentration) by turning into red non-motile encysted cell enclosed with a thick membrane**

The red colour is due to the accumulation of a ketocarotenoid called ASTAXANTHIN (1-5% in dry biomass)

Natural astaxanthin is the most powerful carotenoid antioxidant and *H. Pluvialis* is one of its major producer



While **natural astaxanthin** contains **100% 3S,3'S** enantiomer, the synthetic molecule contains a combination of three different enantiomers

In addition to the **all-trans compound**, the 9- and 13-cis isomers were found to be the most common configurations in the algal extract

The presence of the **conjugate system** of delocalized π electrons is closely linked to astaxanthin chemical-physical properties: it can **absorb light** and has considerable **antioxidant activity** by eliminating free radicals and inhibiting oxygen radical species

Astaxanthin is mainly found in **mono or diesterified** form with various types of **fatty acids** (C18:3, C18:2, C18:1 and C16:0)

Natural astaxanthin is accumulated in **lipid vesicles** composed by triacylglycerols, whose chemical profile is similar to that of astaxanthin esters and can be responsible for up to 40% **of the biomass weight**

H. pluvialis synthesises a mixture of secondary **carotenoids:** astaxanthin, β-carotene, cantaxanthin and lutein

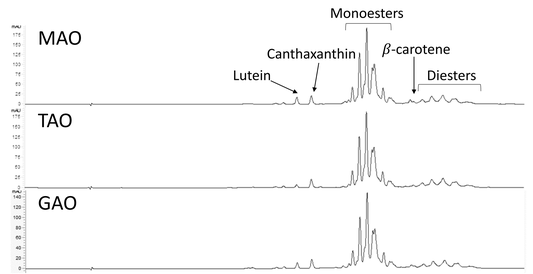
HYDROPHOBIC DEEP EUTECTIC SOLVENTS

- New generation of solvents. Presented in the literature for the first time in 2015
- Mixture of two or more substances, liquid at room temperature, immiscible with water
- Entropy of mixing, van der Waals interactions, and hydrogen bonding plays a role in the formation of DESs.
- They are non-volatile: cannot be separated from the compound they solubilize

DES (molar ratio)	Acronym
Menthol : oleic acid (2:1)	MAO
Thymol : oleic acid (3:1)	TAO
Geraniol : oleic acid (13:1)	GAO

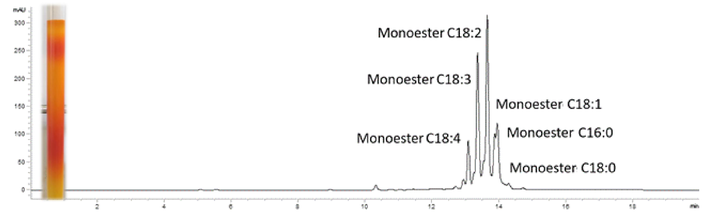
Hydrophobic DES were prepared based on oleic acid mixed with different components (menthol, thymol, and geraniol). It's important to underline that these are natural and edible substances because when a bioactive compound is extracted with a solvent inseparable from it, **the solvent itself should be safe** and sinergic (or neutral) for the bioactive compound

QUALITATIVE ANALYSIS OF THE EXTRACTS



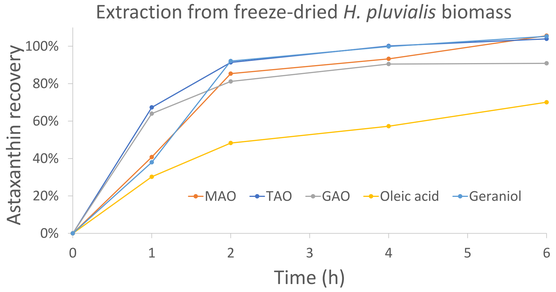
Analysis HPLC-UV/Vis, λ= 470

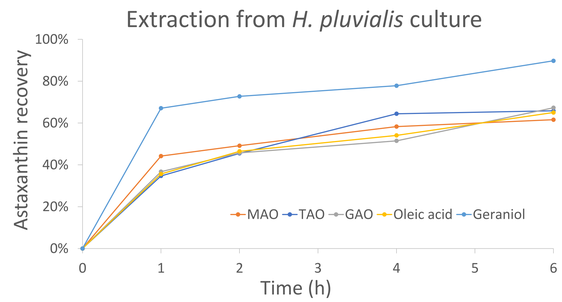
HPLC-analysis showed no significant difference in the carotenoids profile obtained with the three DES. As expected astaxanthin monoester is the main form of astaxanthin biosynthesized by *H. pluvialis* (ratio mono-diesters 3.5:1)

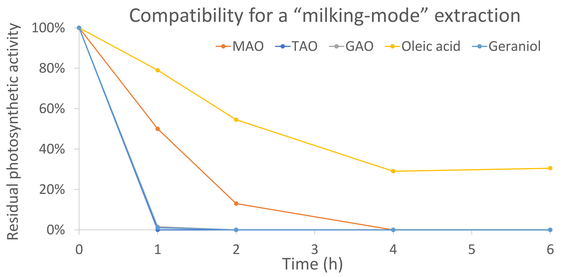


An **astaxanthin monoester** enriched fraction was isolated through flash chromatography. HPLC-MS characterization allowed to determine the fatty acid composition of the monoesters, mainly composed by C18 unsaturated compounds

KINETICS OF EXTRACTION and QUANTITATIVE ANALYSIS OF THE EXTRACTS

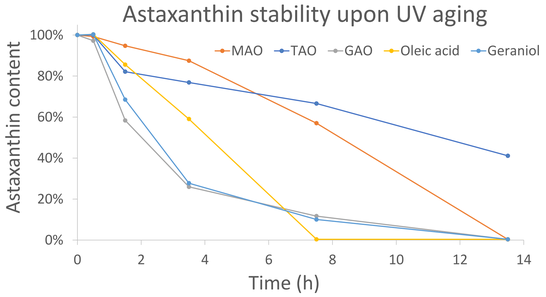






The hydrophobic DES and the respective liquid components (oleic acid and geraniol) were applied to both **freeze-dried biomass and *H. pluvialis* cultures** exploiting their high affinity for hydrophobic molecules such as astaxanthin and their extraction abilities were evaluated

It should be noted that the liquid-liquid extraction from the algal culture exploits the water immiscibility of these solvents to remove the need of harvesting and drying the microalgal culture, known to have a **large impact on the overall energy consumption and economics of the extraction process**

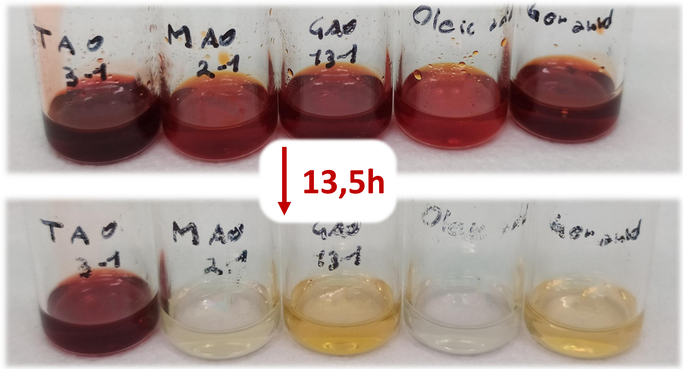


The algal vitality was analysed measuring the **residual photosynthetic efficiency** after the extraction at specific time frames to verify the algal-compatibility of these hydrophobic solvents with the aim of maintaining *H. pluvialis* cells alive and **reusable for a continuous production of astaxanthin**, developing a "milking-mode" extraction

The results clearly demonstrated that preserving the viability of algal cells after the contact with solvents is even more challenging than the extraction process. The algal cell mortality could be related to the toxicity towards algae of each terpene

AGING TEST OF THE EXTRACTS

Because of its well-known instability, astaxanthin tends to easily degrade due to heat, oxygen and light exposure. The potential of DES to stabilize astaxanthin in the extracts was therefore evaluated under controlled "aging" conditions (light and time). All samples with the exception of TAO extract, showed complete degradation of astaxanthin at the end of the study. This result demonstrates the superior potential of TAO to enhance the stability of astaxanthin, it can therefore be considered a **THEDES**, therapeutic DES.



Bioresour. Technol., 2019, 288, 121606; ACS Sustainable Chem. Eng. 2020, 8, 10591−10612; ACS Sustain. Chem. Eng., 2020, 8, 2246−2259; Anal Bioanal Chem (2009) 395:1613–1622; ACS Sustainable Chem. Eng. 2018 6 (8), 10355-10363